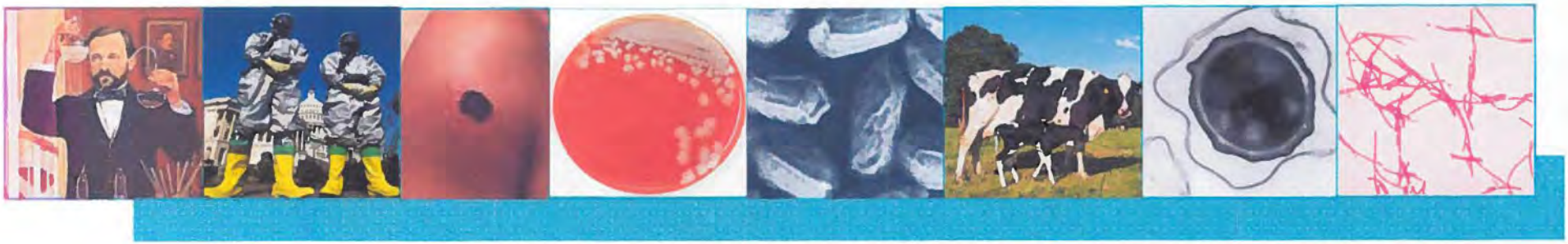

MASTER'S ORAL PRESENTATION (June 19th, 2015):

« Inhibiting inosine hydrolase and alanine racemase to enhance the germination of Bacillus anthracis Sterne spores: potential spore decontamination strategies »

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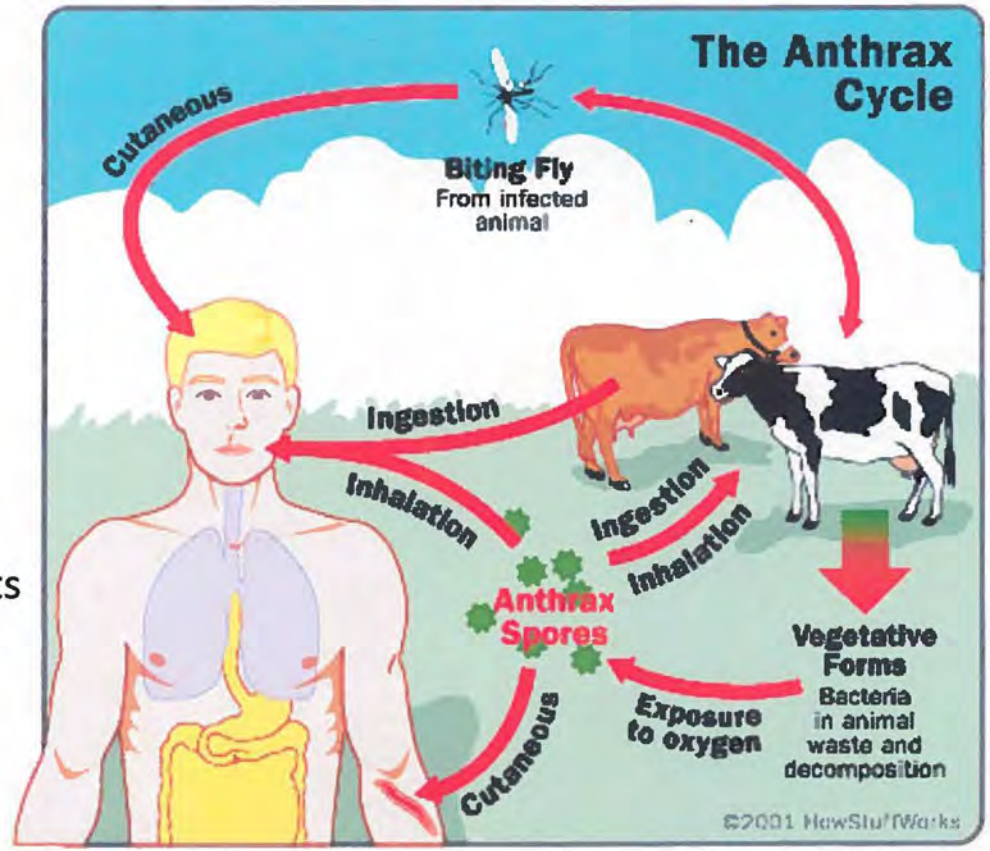
Anthrax Background

- Caused by a gram-positive spore forming rod.
- Important veterinary disease as herbivores may be prone to the disease if they feed in 'anthrax zones'
- Accidentally in Humans
- Natural reservoir is soil
- Anthrax Disease Cycle:

- animals infected by soilborne spores in food and water or bites from certain insects
- Humans can be infected when in contact with flesh, bones, hides, hair or excrement



PR-15-306

Anthrax Background

- **4 forms**: cutaneous and inhalational most common.

Cutaneous *Inhalation* *Ingestion* *Injection*

- **Concern for Biodefense Community: Intentional or Accidental release of spores**
- ***Why?*** Anthrax spores are easily found in nature, can be produced in a lab, and can last for a long time in the environment.
- ***How?*** Can be released easily and quietly. Nobody is able to see, smell, or taste them. Signs and symptoms are non-descript flu-like symptoms making rapid diagnosis difficult
- Decontamination difficult, expensive and with toxic/corrosive effects to the environment and other sensitive materials.
- Example: 2001 Anthrax letters US Postal System, October, 2001.
  - 22 cases, 11 inhalational, 5 deaths
  - $650 million and took more than three years

PR-15-306

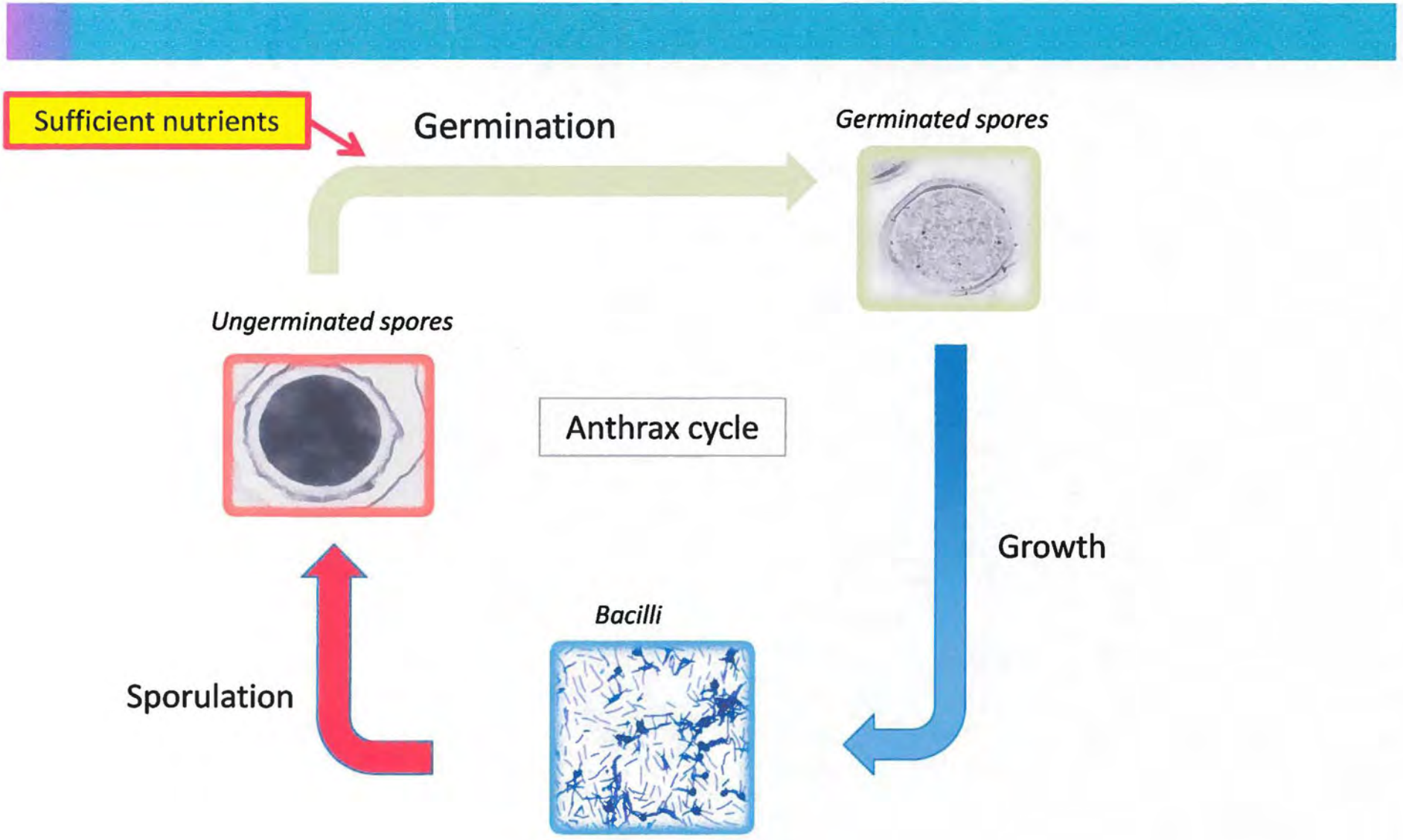
Decontamination

- US Government Priority after 2001
- Current decontamination methods include:
  - Burn or bury animal carcasses
  - Treat soil with 5% lye, quicklime, or bleach (sodium hypochlorite)
  - High-efficiency particulate arrestance vacuuming (source reduction)
  - Liquid antimicrobials (non-porous surfaces)
  - Fumigation (chlorine dioxide, vaporous hydrogen peroxide)

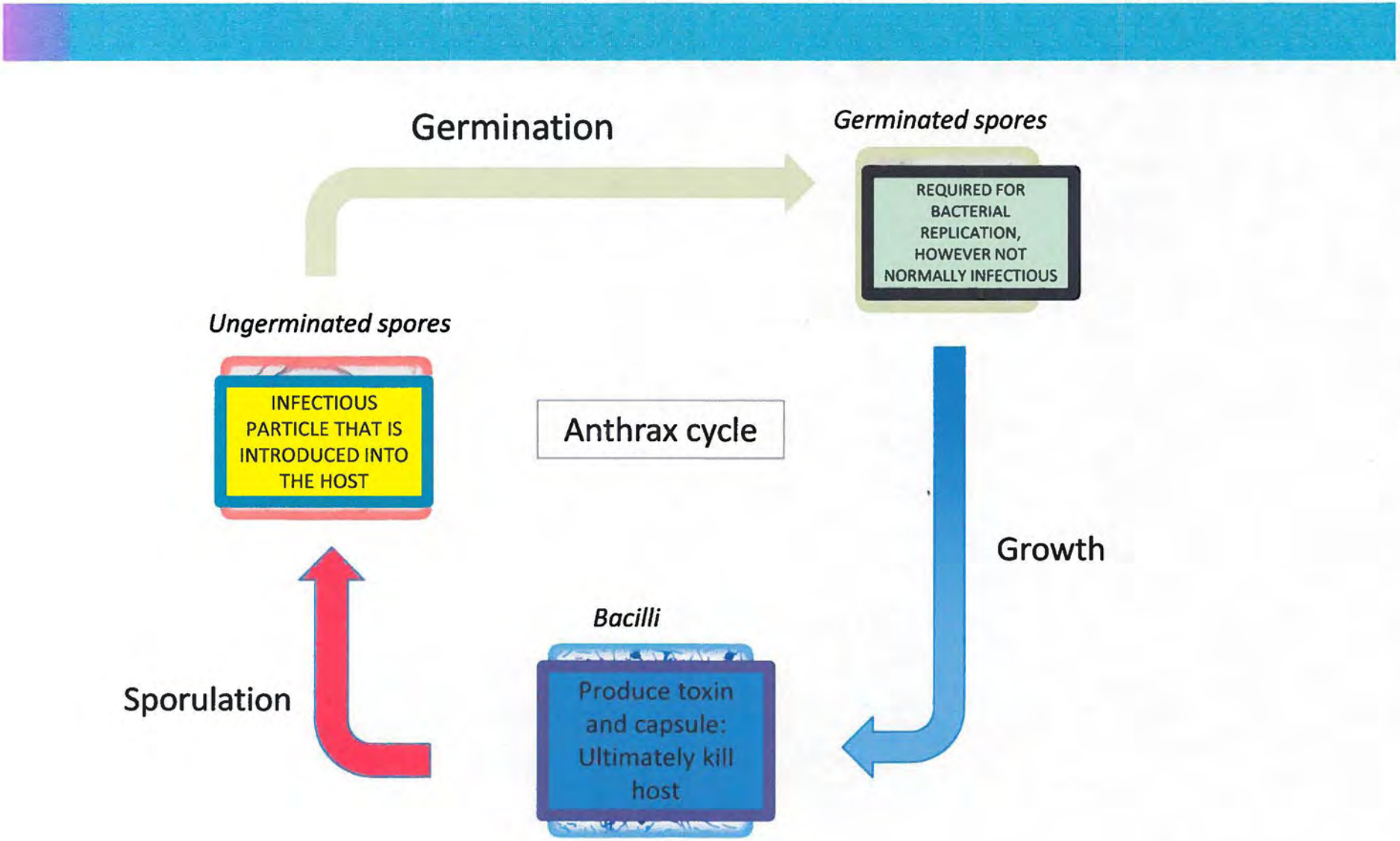
- Decontamination objectives: be EASIER, SAFER, and CHEAPER

  Inducing spore germination should make resulting bacteria much more susceptible to decontamination methods and will be less hazardous to first responders.

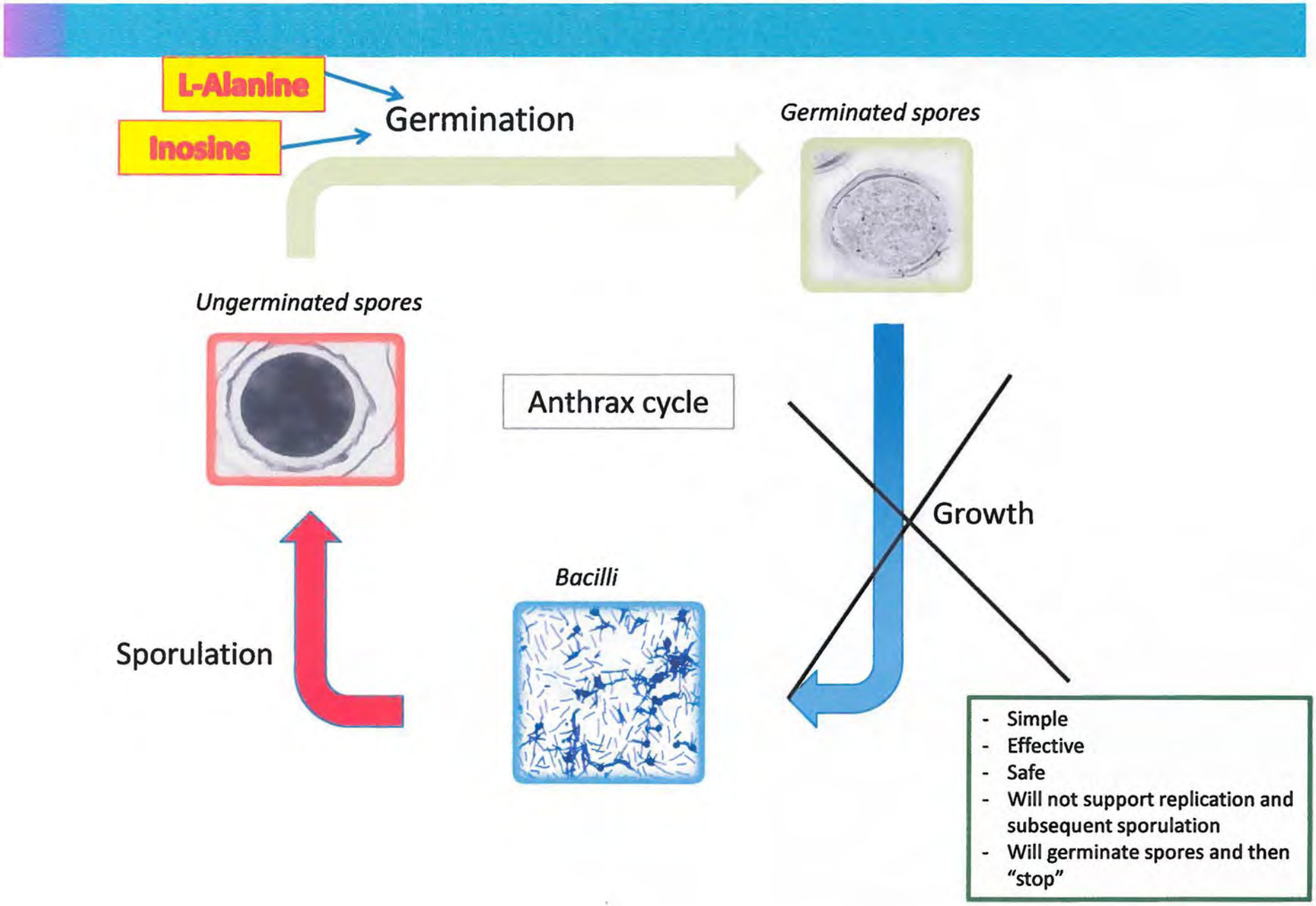
Bacillus anthracis Cycle



Bacillus anthracis Cycle

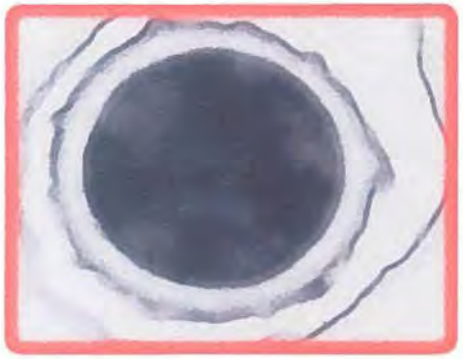


In vitro germination induction by Al



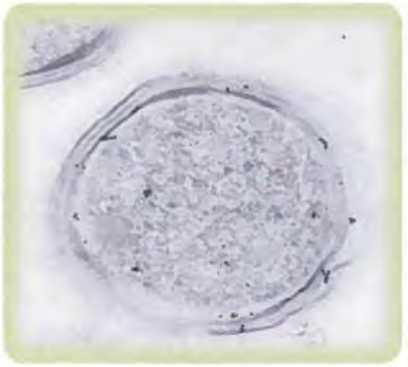
Interest of AI inducted germination

Ungerminated spores



Germination

Germinated spores



Resistant

- to desiccation
- to most of desinfectant
- to antibiotics
- to heating
- to host immune response

Sensitive

- to desiccation
- to most of desinfectant
- to heating
- to antibiotics
- to host immune response

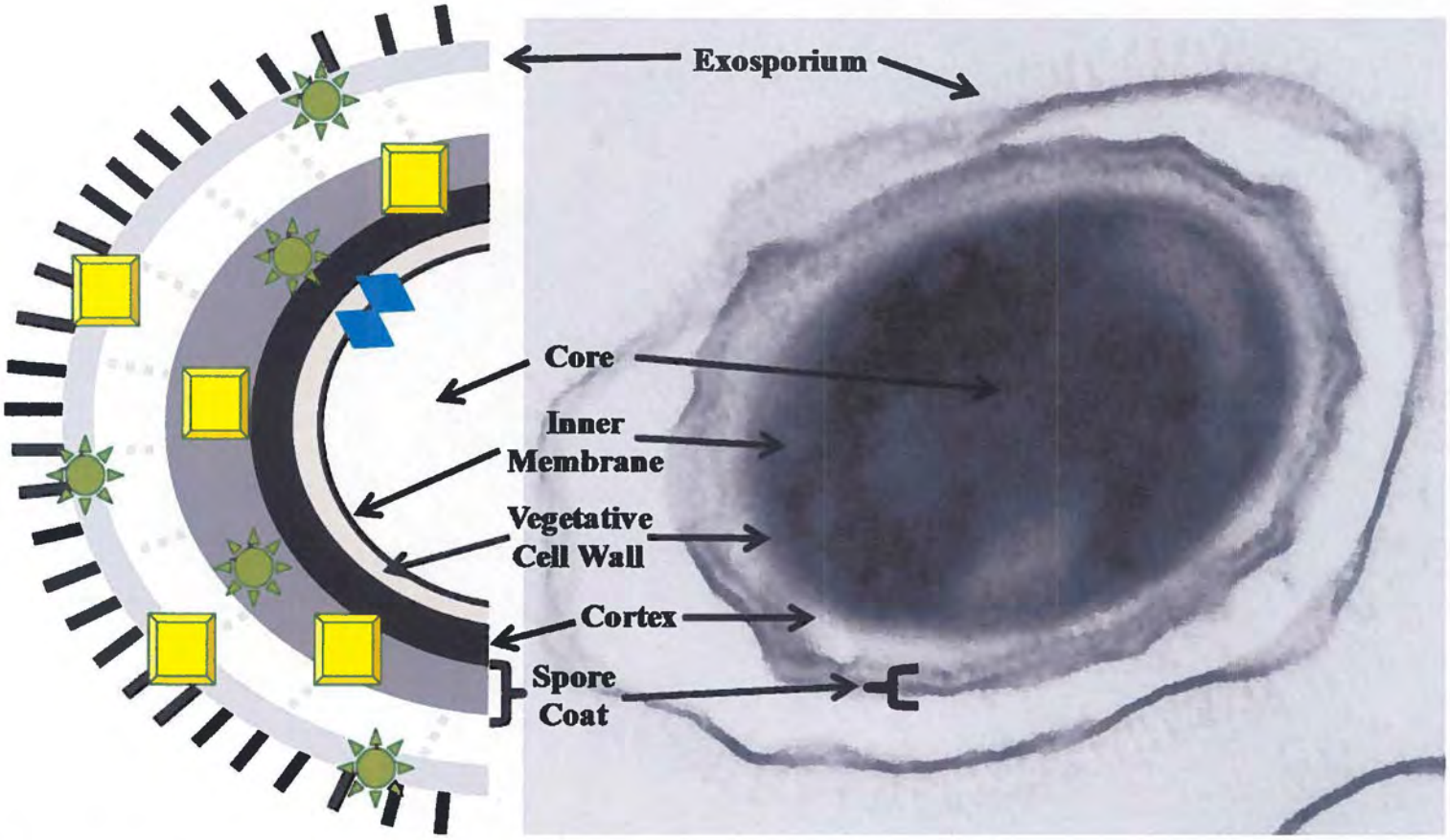
In vitro alanine and inosine germination pathways

- **L-alanine**
  - L-amino acid
  - Can acts alone
  - Action on specific germinant receptors (*ger*R)
  - Action on enzyme alanine racemase (Alr)

  - Alr inhibits by the antibiotic D-cycloserine (Gould 1968, Omotade *et al.*, 2013)

- **Inosine**
  - purine nucleoside
  - Co-germinant only in *Bacillus anthracis*
  - Action on specific germinant receptors (*ger*I, *ger*Q and *ger*R)
  - Action on Inosine uridine nucleoside Hydrolase (IunH)

Localization of enzymes



Alanine racemase

Inosine uridine nucleotide hydrolase

Germinant receptor

Objectives

- Test the impact of the inactivation of two germination-inhibiting enzymes, alanine racemase and inosine hydrolase on the alanine and inosine induced germination:
  - using a *iunH* gene deletion
  - by D-cycloserine treatment
- in order to identify new strategies for an efficient decontamination.

BIOHAZARD

Biosafety Level 2

AUTHORIZED PERSONNEL ONLY

Material

- Attenuated *B. anthracis* strain Sterne (pXO1+, pXO2-): veterinarian vaccinal strain. Lost its ability to produce a capsule.
- Inosine hydrolase (IunH) defective mutant of Sterne strain with kanamycin insertion (Sterne *iunh*::Ω-kan-2) from Biology Department at Louisiana Tech University, Ruston, LA.

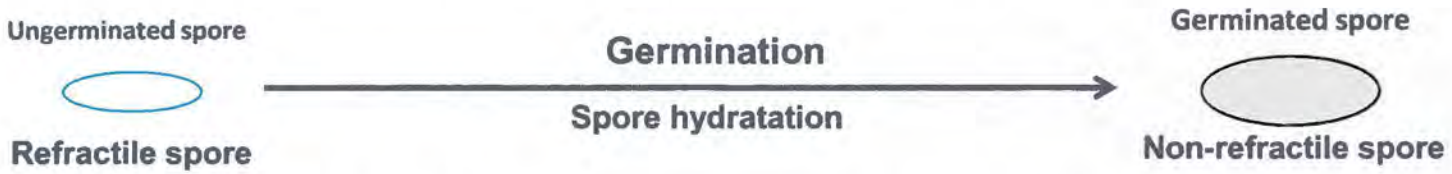
Methods

In vitro detection of spores germination induced by AI

- ***Heat resistance assay***

  Once spores germinate they become sensitive to elevated temperatures, thus a difference in viable colony forming unit/ml (cfu/ml) in samples that were heated versus samples that were not subjected to heat treatment, reflects the amount of germination induced.

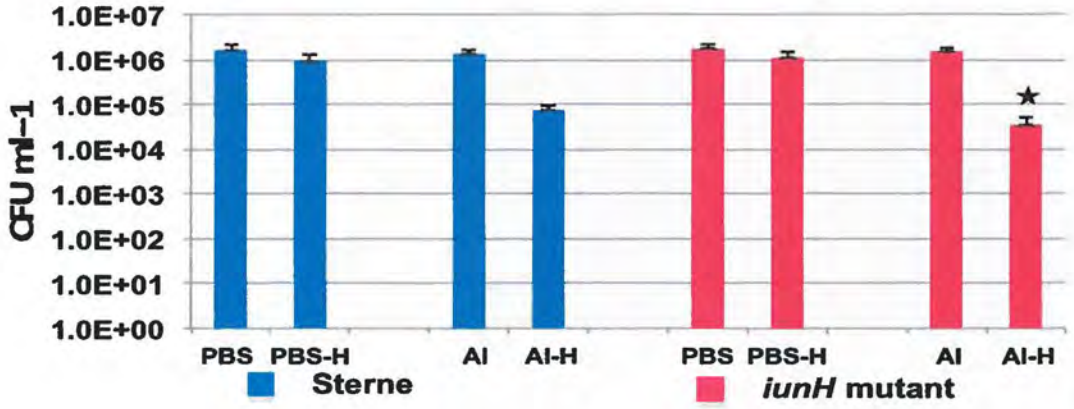
- ***Loss of optical density:*** spectrophotometric determination of germination rate based on alterations in spore refractility. During the process of germination, spore releases its large pool of Ca^{2+}-dipicolinate stored in the core, and becomes partially rehydrated through an influx of water.



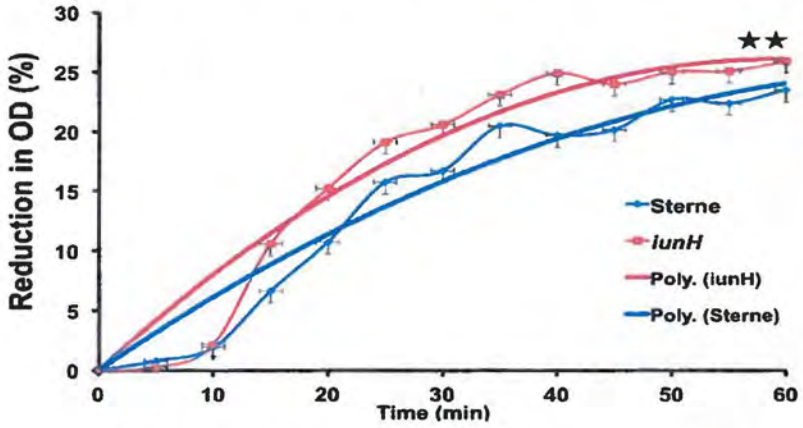
- ***Fluorescence spectrophotometry*** (Welkos *et al.*, 2004): increase in fluorescence of spores with time during their incubation in germination medium containing a fluorescent nucleic acid-binding dye which stained germinated *B. anthracis* but not ungerminated spores.

Results: inosine hydrolase inhibition

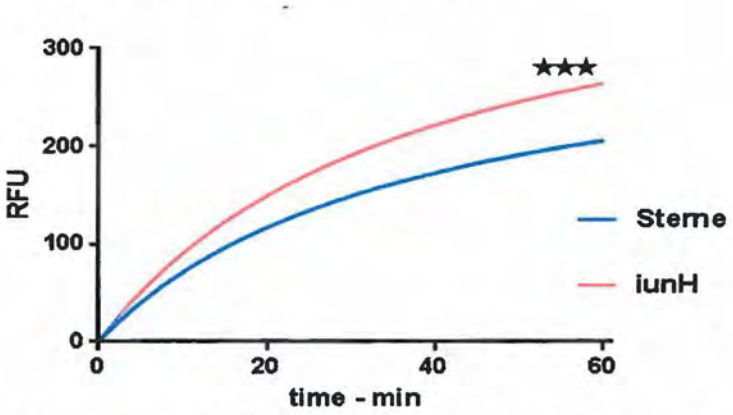
(a) Heat resistance assay



(b) Spectrophotometer assay

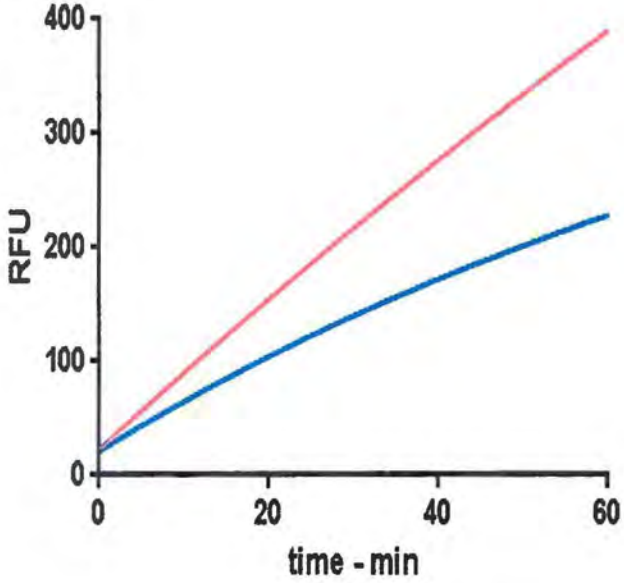


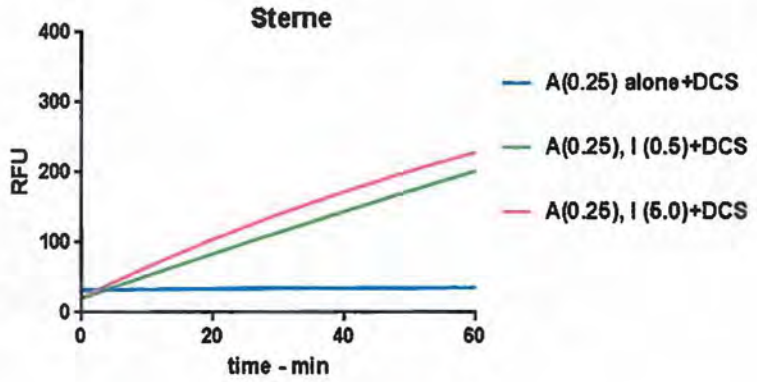
(c) Fluorescent assay

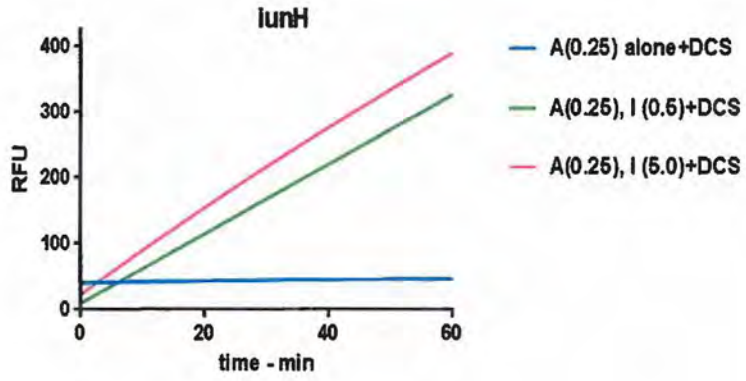


Spores deficient in the inosine hydrolase (encoded by *iunH*) germinate more rapidly than wild-type spores

Results: both enzymes inhibition



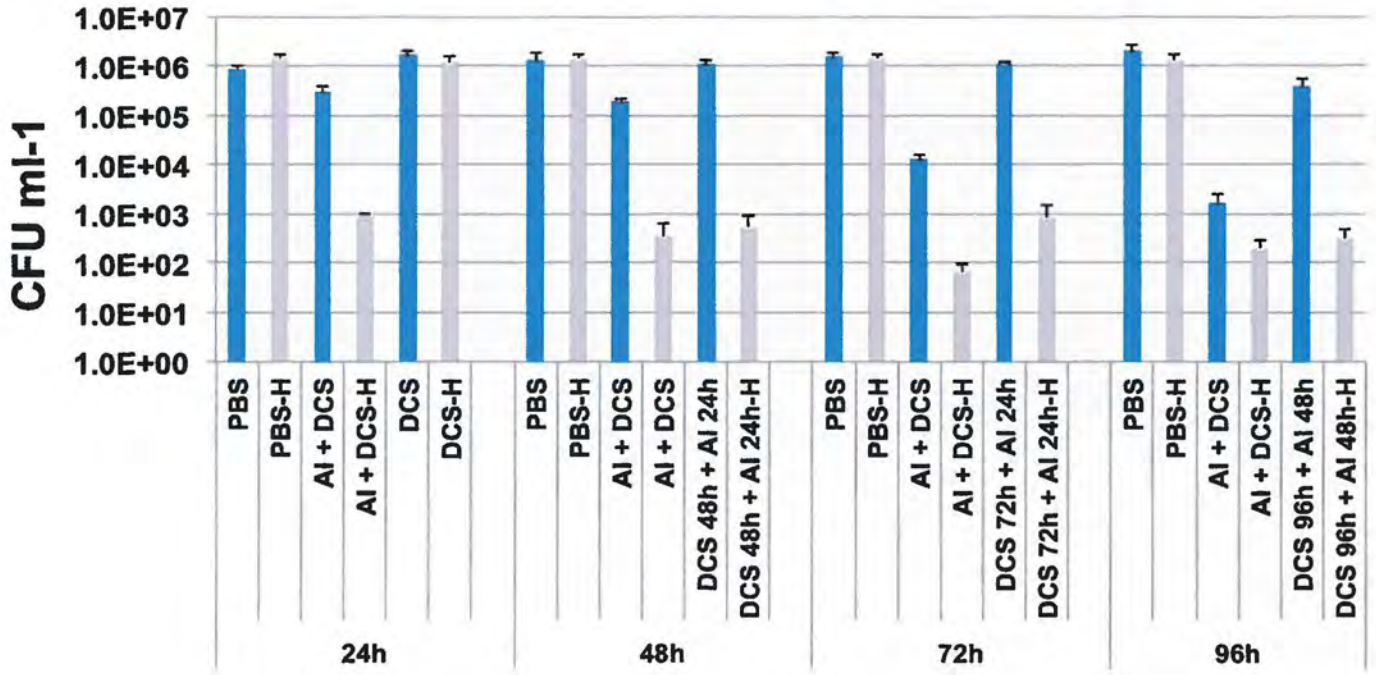




Germination rate of *iunH* mutant spores initiated by L-alanine and inosine in presence of DCS 10 mmol $l^{-1}$was significantly greater than the germination rate of the wild-type spores under same conditions (p=0.0001)

Results: interest of a 24h DCS pretreatment in Sterne

- Previously demonstrated that DCS is dose and time dependant (Omotade *et al.*, 2013)



Concomitant delivery of DCS with germinant solutions is more beneficial to wide-area decontamination efforts that pretreatment with DCS followed by germinant solutions

Conclusion

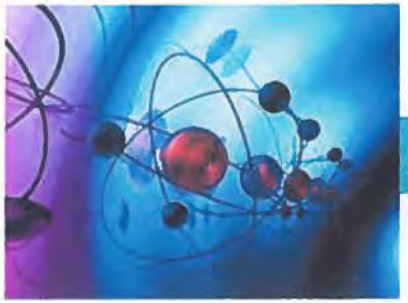
Interest in context of novel decontamination strategies

- Increase of the germination rate induce:
- By inhibiting Alr and IunH separately
- By inhibiting the both concommitantly (*iunH* mutant spores positively affected by the block of Alr)

- Better understanding and manipulating spore germination.

- Induction of the transition from highly resistant ungerminated spores to much more susceptible and less virulent germinated spores.

- Strengthens the early work published in 2013 and 2014 showing that spore germination rates are augmented potentially improving decontamination strategies.



Prospect for the future

- Optimize the L-alanine concentration in addition to the inosine concentration in presence of DCS

- Test potential inhibitors of *B. anthracis* inosine hydrolase and prove that such enzymatic inhibitors could be used in conjunction with DCS to facilitate more efficient and environmentally friendly surface decontamination of *B. anthracis* spores.

- Test current decontamination methods after germination induction by inhibiting both AIr and IunH

USAMRIID BACTERIOLOGY DIVISION

- acknowledgements

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